Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling

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Wnt signals regulate differentiation of neural crest cells through the β-catenin associated with a nuclear mediator of the lymphoid-enhancing factor 1 (LEF-1)/T-cell factors (TCFs) family. Here we show the interaction between the basic helix–loop–helix and leucine-zipper region of microphthalmia-associated transcription factor (MITF) and LEF-1. MITF is essential for melanocyte differentiation and its heterozygous mutations cause auditory–pigmentary syndromes. Functional cooperation of MITF with LEF-1 results in synergistic transactivation of the dopachrome tautomerase (DCT) gene promoter, an early melanoblast marker. This activation depends on the separate cis-acting elements, which are also responsible for the induction of the DCT promoter by lithium chloride that mimics Wnt signaling. β-catenin is required for efficient transactivation, but dispensable for the interaction between MITF and LEF-1. The interaction with MITF is unique to LEF-1 and not detectable with TCF-1. LEF-1 also cooperates with the MITF-related proteins, such as TFE3, to transactivate the DCT promoter. This study therefore suggests that the MITF/TFE3 family is a new class of nuclear modulators for LEF-1, which may ensure efficient propagation of Wnt signals in many types of cells.

Keywords: dopachrome tautomerase/LEF-1/melanocyte/MITF/Wnt

Introduction

Transcription factors containing a basic helix–loop–helix and leucine-zipper (bHLH/LZ) structure play critical roles in regulatory networks of many developmental pathways, cell growth and differentiation (Murre et al., 1994). The basic region permits the bHLH/LZ proteins to bind to the E box motif (CANNTG) and the HLH/LZ region allows these proteins to form homodimers and/or heterodimers. Microphthalmia-associated transcription factor (Mitf), encoded by the mouse Mitf gene, belongs to an evolutionarily ancient family of the bHLH/LZ proteins (Atchley and Pritch, 1997). Mitf plays an important role in the differentiation of various cell types, including melanocytes of neural crest origin, optic cup-derived retinal pigment epithelium (RPE), and bone marrow-derived mast cells and osteoclasts (Hodgkinson et al., 1993). In addition, mutations in the Mitf gene, the human counterpart of the Mitf gene, are associated with dominantly inherited auditory–pigmentary syndromes, which are characterized by sensorineural hearing loss and abnormal pigmentation of the hair and skin (Tassabehji et al., 1994; Nobukuni et al., 1996; Amiel et al., 1998).

Recent studies have revealed the isomeric multiplicity of Mitf/Mitf, which could account in part for various phenotypic consequences of Mitf/Mitf mutations (reviewed in Yasumoto et al., 1998; Shibahara et al., 1999). In fact, Mitf is composed of at least five isoforms with distinct N-termini, Mitf-M, -H, -A, -B and -C (Amae et al., 1998; Mochi et al., 1998; Fuse et al., 1999; Udono et al., 2000). These isoforms share the entire downstream region, including the transcriptional activation domain and the bHLH/LZ domain. In addition, Mitf shares significant amino acid sequence similarity with transcription factors such as TFE3, TFEB and TFE, especially in the bHLH/LZ region (Beckmann et al., 1990; Carr and Sharp, 1990; Yasumoto and Shibahara, 1997). Mitf-M is expressed specifically in melanocytes and melanoma cells, although other isoforms are expressed in various tissues and cultured cell lines (Amae et al., 1998; Yasumoto et al., 1998; Fuse et al., 1999). In cultured cells, Mitf-M transactivates the melanogenesis enzyme genes, tyrosinase and tyrosinase-related protein-1 (TRP-1), through the cis-acting DNA elements containing a CATGTG motif, such as M box (Yasumoto et al., 1994, 1995, 1997; reviewed in Goding, 2000).

Wnt, a group of secretory signaling molecules, evokes a signal to regulate melanocyte differentiation (Patapolvanich and Reichardt, 2000). The binding of Wnt to its receptor Frizzled leads to inactivation of glycogen synthase kinase-3β (GSK3β), followed by the accumulation of β-catenin and its translocation to the nucleus. Lymphoid-enhancing factor 1 (LEF-1)/T-cell factor (TCF) transcription factors can bind to the β-catenin and the complexes formed transactivate the target genes (Cadigan and Nusse, 1997; Barker et al., 2000). A recent study has shown that injection of β-catenin mRNA into zebrafish embryos increases the population of pigment cells of the neural crest origin (Dorsky et al., 1998). Direct gene transfer of Wnt1 or β-catenin to mouse neural crest cells resulted in melanocyte expansion and differentiation (Dunn et al., 2000). We have shown that exogenously added Wnt-3a protein to cultured murine melanocytes increased the expression of endogenous MITF mRNA and transactivated the melanocyte-specific M promoter of the Mitf gene through the LEF-1 site (Takeda et al., 2000). These results suggest that the Wnt signaling pathway regulates the differentiation of melanocytes from neural crest cells by activating the M promoter. In fact, selective
requirement of Mitf-M for melanocyte development was verified by the molecular analysis of recessive black-eyed white Mitf<sup>mi-mw</sup> mice that are deficient in Mitf-M expression (Yajima et al., 1999). Moreover, in zebrafish embryos, Wnt signaling directly activates nacre, a zebrafish MITF homolog, which is required for the formation of neural crest-derived pigment cells (Dorsky et al., 2000). Expression of dopachrome tautomerase (DCT) is almost entirely absent from neural crest cells in nacre<sup><sup>−/−</sup></sup> embryos, and conversely, misexpression of nacre induced ectopic expression of DCT in wild-type and mutant embryos (Lister et al., 1999). In mice, the DCT gene has been established as an early melanoblast marker (Steel et al., 1992). Taken together, these results suggest that the DCT gene may be a downstream target of Wnt signaling.

Here we show the functional interaction of MITF-M and LEF-1 using the DCT gene promoter, which represents a novel mechanism by which Wnt signaling leads to transcriptional activation of target genes.

**Results**

**Transactivation of the DCT promoter by MITF-M and LEF-1**

Expression of LEF-1 mRNA was analyzed in various cell types by RT–PCR (Figure 1A). LEF-1 mRNA is expressed in melanin-producing cells, such as melanocytes, melanoma cells and RPE, and in other cell types examined, but not in HeLa cells. The lack of LEF-1 expression in HeLa cells is consistent with the previous report by Giese et al. (1995). In contrast, TCF-1 mRNA was detected in all cell types, but its expression level seems to be lower in HeLa cells. We therefore performed transient cotransfection assays in HeLa cells to analyse the effect of LEF-1 on the human DCT promoter. Both LEF-1 and MITF-M showed
no or only marginal effects on DCT promoter activity (Figure 1B), despite the fact that the promoter contains the M box, which is bound by MITF-M (Yasumoto et al., 1997), and a potential LEF-1-binding site in DDE1 (Amae et al., 2000). Unexpectedly, synergistic transactivation of the DCT promoter was observed when LEF-1 and MITF-M were coexpressed. Deletion studies suggest that the two separate regions, -268 to -222 and -185 to -161, are involved in the transactivation by MITF-M and LEF-1. Moreover, the mutation at the M box (-138 to -128) reduced the degree of transactivation by ~2- to 3-fold.

To explore the role of Wnt signaling in the observed activation of the DCT promoter, we analyzed the effects of LiCl, an inhibitor of GSK3β, which mimics Wnt signaling (Klein and Melton, 1996). For this series of experiments, a mouse melanocyte cell line, melan-a, was used (Bennett et al., 1987), because melan-a cells are able to respond to Wnt signaling (Takeda et al., 2000) and express TCF-1 and LEF-1 mRNA endogenously (Figure 1A). Treatment with LiCl resulted in activation of the DCT promoter through a cis-acting region (-268 to -222) that is also required for the transactivation by MITF-M and LEF-1 (Figure 1B and C). Notably, no activation by LiCl was detected with a construct pHDTL22, carrying the 222-bp promoter region, unlike the effect of coexpression of MITF-M and LEF-1.

We also attempted to identify the core sequence of another region (~185 to -161) (Figure 1B) by using four pHDTL12-derived constructs carrying various base changes that cover the entire 25-bp region. However, we were unable to detect any significant effects of the base changes on the promoter activation by MITF-M and LEF-1. The 25-bp element lacks the E box and the potential binding sites for LEF-1/TCFs, and it is not required for activation by LiCl (Figure 1C). This 25-bp element may be less physiologically important, and its function was detected only when the identified cis-regulatory region (-268 to -222) is deleted, as in the case of pHDTL22 (Figure 1B and C).

**A cis-acting element that is required for activation by MITF-M and LEF-1**

We then identified the cis-acting region (~249 to ~233) that is required for the transactivation by cooperation of MITF-M and LEF-1 (Figure 2A and B). This 17-bp region contains a motif similar to a cAMP-responsive element.
(CRE), termed the CRE-like motif (Bertolotto et al., 1998), but does not contain the potential E box or the binding sites for LEF-1/Tcf. This region is also required for activation by LiCl in melan-a cells (data not shown). Base changes were then introduced into the 17-bp segment of construct pHDL12 (Figure 2B and C). Synergistic effects of MITF-M and LEF-1 were detected with a mutant construct carrying the intact CRE-like motif but reduced by ∼2.5- to 4-fold with the constructs carrying the base changes at the CRE-like motif. Thus, the 5′-TGA-3′ and 5′-GTC-3′ sequences of the CRE-like motif are required for the activation by MITF-M and LEF-1.

We then analyzed whether nuclear proteins bind the 17-bp segment (−249 to −233) by electrophoretic mobility shift assays (EMSAs) (Figure 2D). A synthetic primer DTPR2 of 20 bp was bound by nuclear extracts prepared from the HeLa cells expressing LEF-1 and MITF-M or the mock-transfected cells, and no difference in the DNA-binding activity was detected between these two nuclear extracts (lanes 2 and 3, 14 and 15). Thus, the detected protein-DNA complex did not contain LEF-1 and MITF-M, which are deficient in HeLa cells. The DNA-binding activity was competed for by a competitor DTPR2, DTPR1 or DTM6 (lanes 4–7 and 12), but not by DTM7 (lanes 8 and 9), DTM8 (lanes 10 and 11), a synthetic CRE (lanes 16 and 17), a LEF-1-binding site (lanes 18 and 19) or the M box (data not shown). Thus, a third factor specifically binds the 12-bp element that is shared by DTPR2 and DTPR1, and is different from MITF-M and LEF-1. Similar DNA-binding activity was also detected in melanoma nuclear extracts (data not shown). It is noteworthy that the DNA-binding activity was not competed for by a synthetic CRE, suggesting that this CRE-like motif-binding protein may be different from CRE-binding proteins.

**Functional cooperation between MITF-M and LEF-1**

We next searched for the LEF-1-binding site in the DCT promoter region. The deletion study suggested that the downstream region (−161 to −56) is also required for the transactivation by LEF-1 and MITF-M (data not shown). Consequently, using several synthetic fragments covering the relevant region as EMSA probes, we have identified the CTTTGGG sequence (−143 to −137) as a LEF-1-binding site (Figure 3A); namely, this element was specifically bound by the full-length LEF-1 fused to glutathione S-transferase (GST). The LEF-1-binding activity of this element was also confirmed with nuclear extracts of HeLa cells expressing LEF-1 (data not shown).

To search for a domain of LEF-1 that is required for transactivation of the DCT promoter, we first analyzed the involvement of the N-terminus of LEF-1, which contains the β-catenin-binding domain (amino acid residues 2–37) (Behrens et al., 1996; Molenaar and van de Wetering, 1996). The degree of transactivation was reduced when MITF-M was coexpressed with dominant-negative LEF-1.
(DNLEF-1), which lacks the β-catenin-binding domain (Figure 3B). It is noteworthy that DNLEF-1 shows a weak but significant activation of the DCT promoter with MITF-M (~5-fold in Figure 3B). Moreover, β-catenin alone did not noticeably activate the DCT promoter (data not shown), but co-expression of β-catenin with LEF-1 and MITF-M significantly enhanced the synergistic activation of the DCT promoter by LEF-1 and MITF-M (4.13 ± 0.40-fold). Thus, β-catenin is involved in the efficient cooperation of LEF-1 with MITF-M on the DCT promoter.

The C-terminal portion of LEF-1 was also analyzed (Figure 3B). LEF-1ΔC lacks the C-terminal 25 amino acid residues (positions 375–399), and LEF-1ΔC2 lacks the C-terminal 15 residues (385–399) but retains the nuclear
localization signal (NLS) (Figure 4A). Surprisingly, LEF-1ΔC2 by itself transactivated the $DCT$ promoter and the degree of transactivation by LEF-1ΔC2 was further enhanced by MITF-M (Figure 3B). LEF-1ΔC2, fused to enhanced green fluorescent protein (EGFP), was specifically targeted to the nucleus (Figure 3C). In contrast, no activation was detected with LEF-1ΔC, which lacks the NLS, despite its noticeable expression in the nucleus. In fact, ~34% of expressed tagged-LEF-1ΔC were detected in the nuclear extracts (Figure 4B). Thus, the C-terminal domain of 15 residues, deleted in LEF-1ΔC2, appears to repress the function of LEF-1. In addition, the 10 residues (positions 375–384), which are deleted in LEF-1ΔC, are required for the functional cooperation with MITF-M or with certain endogenous coactivators. The lack of transactivation by LEF-1ΔC is consistent with the crucial role for the NLS, which is known to be responsible for DNA binding (Carlsson et al., 1993).

**Physical interactions between MITF-M and LEF-1**

We next examined the physical interaction between MITF-M and the C-terminus of LEF-1 by pull-down assays. Both LEF-1 and TCF-1B possess an identical HMG box and share a similar NLS (van de Wetering et al., 1996) (Figure 4A). The mobility of c-Myc-tagged LEF-1 was similar to that of the endogenous c-Myc protein, and was detected as enhanced signals in the nuclear extracts (Figure 4B, lanes 1–3). Tagged LEF-1 mutants, including LEF-1ΔC lacking the NLS, were detected in the nuclear extracts at the expected sizes (lanes 4–6). LEF-1 and DNLEF-1 were also detected in the bound fraction (lanes 8 and 9). Thus, LEF-1 and DNLEF-1 can bind MITF-M, indicating that the β-catenin interaction domain is not required for the interaction with MITF-M. Moreover, MITF-M was able to bind LEF-1ΔC2 retaining the NLS (lane 10), but not LEF-1ΔC lacking the NLS (lane 11).

To confirm the functional significance of the C-terminus of LEF-1, we analyzed the effects of TCF-1B on the $DCT$ promoter (Figure 4C). Interestingly, p45-TCF-1B, a full-length isoform of TCF-1, transactivated the $DCT$ promoter, but no enhancing effects were observed when coexpressed with MITF-M. A dominant-negative isoform of TCF-1B (DNLEF-1B), p33-TCF-1B, lacking the β-catenin-binding domain (van de Wetering et al., 1996), showed no noticeable effects on the $DCT$ promoter. These results suggest that the functional cooperation with MITF-M is unique to LEF-1 and is not a general feature of TCFs. In addition, TCF-1B transactivates the $DCT$ promoter by cooperating with β-catenin but not with MITF-M. Accordingly, we analyzed the functions of LEF-1–TCF-1B chimeric proteins. LEF-1RS contains the NLS of TCF-1B instead of the LEF-1 NLS, and LEF-1T contains the C-terminus of TCF-1B. The function of LEF-1RS is indistinguishable from that of LEF-1, despite the two amino acid differences in their NLSs. Interestingly, like p45-TCF-1B, LEF-1T transactivated the $DCT$ promoter, but unlike p45-TCF-1B, LEF-1T did cooperate with MITF-M. Thus, the C-terminus of LEF-1, deleted in LEF-1T, may function as a repression domain, which is consistent with the activation of the $DCT$ promoter by LEF-1ΔC2 (see Figure 3). In addition, the NLS of TCF-1B is able to mimic the function of the LEF-1 NLS, indicating that the interaction with MITF-M depends on the middle portion of LEF-1, located between the β-catenin interaction domain and the HMG box.

**Functional difference between LEF-1 and TCF-1B**

We analyzed the effect of a dominant-negative form of LEF-1 or TCF-1B on the basal promoter and the LiCl-mediated activation of the $DCT$ promoter in melan-a cells (Figure 5). In this melanocyte cell line, MITF-M, LEF-1 or TCF-1B only marginally transactivated the $DCT$ promoter activity (~2-fold). These results suggest that MITF-M and LEF-1 could interact with endogenous LEF-1 and MITF-M, respectively. Interestingly, DNLEF-1B, but not DNLEF-1, significantly reduced the $DCT$ promoter activity (~3-fold). Thus, TCF-1B may compete for the binding site with endogenous LEF-1. Moreover, remarkably, only DNLEF-1B reduced the LiCl-mediated activation of the $DCT$ promoter. These results suggest that both LEF-1 and TCF-1B share the binding sites but the mechanism by which TCF-1B transactivates the $DCT$ promoter is different from that by LEF-1, which is consistent with our proposal that MITF-M is able to interact with LEF-1 and even with DNLEF-1, but not with TCF-1B.

**The bHLH/LZ region of MITF-M as an interacting domain with LEF-1**

We then localized the domain of MITF-M, which is required for the interaction with LEF-1, by pull-down assays (Figure 6A). Tagged LEF-1 was detectable in the fractions bound to truncated MITF-M proteins containing the bHLH/LZ domain (lanes 9–11), whereas no tagged LEF-1 was detected in the fraction bound to GST (lane 8) or truncated MITF-M carrying only its C-terminal region (lane 12). These results indicate that the bHLH/LZ domain of MITF-M is required for the interaction with LEF-1.

To confirm the involvement of the bHLH/LZ region in the interaction with LEF-1 in vivo, we next performed yeast two-hybrid experiments (Figure 6B). LEF-1 was chosen as bait, because a strong transactivation domain is located near the N-terminal region of MITF-M (Sato et al.,
Fig. 6. Interaction of the bHLH/LZ region of MITF-M with LEF-1. (A) In vitro interaction between LEF-1 and the bHLH/LZ domain of MITF. COS-7 nuclear extracts contained endogenous c-Myc (lanes 1–12), as indicated by an arrow. Tagged LEF-1 was detected as enhanced signals in the fractions, bound to truncated MITF-M proteins containing the bHLH/LZ domain (lanes 9–11). An arrowhead indicates the unspecific protein binding (lanes 1–7). (B) Interaction between LEF-1 and the bHLH/LZ region of MITF-M in yeast cells.

1997) and LEF-1 itself does not act as a transcriptional activator in yeast cells (Prieve et al., 1998). When the full-length LEF-1 plasmid was introduced into yeast cells together with the plasmid, carrying a large portion of MITF-M, significant β-galactosidase activity was detected. Similarly, β-galactosidase activity was detected with a plasmid, carrying only the bHLH/LZ region. In contrast, no β-galactosidase activity was detected with the parent vector or the plasmid, containing the C-terminal region of MITF-M. These results support the notion that the bHLH/LZ region of MITF-M is responsible for the association with LEF-1 in vivo.

Effects of mutations in the bHLH/LZ region on the interaction with LEF-1
To confirm the crucial role for the bHLH/LZ region of MITF-M in the interaction with LEF-1, we introduced an Asp222Asn substitution in helix 1 and a Gly244Glu
wild-type and mutant MITF-M proteins in transfected HeLa cells (data not shown).

Unexpectedly, MITF-M<sup>αα</sup> protein and MITF-M<sup>bb</sup> protein showed differential effects on the cooperative activation of the DCT promoter by LEF-1 (Figure 7B). The degree of synergistic activation by LEF-1 and MITF-M<sup>αα</sup> protein or MITF-M<sup>bb</sup> protein was ~25 or 70% of the control value obtained by the combination of LEF-1 and MITF-M, respectively. MITF-M<sup>αα</sup> protein may act on the DCT promoter as a non-DNA-binding cofactor for LEF-1 because it is deficient in the DNA-binding activity. In this context, the α and β mutations did not noticeably impair the in vitro interaction of mutant MITF proteins with LEF-1, as judged by the pull-down assays (data not shown). These results suggest that the mutations may differentially influence the interaction between MITF-M, LEF-1, a CRE-like motif-binding protein, and other factors, such as CBP/p300, which were reported to associate with MITF-M (Sato et al., 1997) (see Figure 9).

The functional consequences of the α and β mutations were also assessed by mammalian two-hybrid assays (Figure 7C). The bHLH/LZ region of MITF-M (residues 138–372; see Figure 6A) was fused to the transactivation domain of VP16 protein, and LEF-1 was fused to the GAL4 DNA-binding domain. A reporter luciferase gene was under the control of the promoter containing five copies of the GAL4 DNA-binding site. The expressed luciferase activities were near the background levels when the MITF–VP16 fusion protein carries the α or β mutation. Thus, these mutations impair the interaction between the bHLH/LZ region and LEF-1 on the GAL4 promoter, probably due to the profound conformational changes of the fusion protein or the lack of the CRE-like motif in the GAL4 promoter.

The bHLH/LZ region as a common interacting domain with LEF-1

To assess whether interaction with LEF-1 is a general feature of bHLH/LZ proteins, we examined effects of other bHLH/LZ transcription factors, including TFE3, TFEC and c-Myc (Watt et al., 1983) (Figure 8A). MITF-A, an isoform of MITF-M, possesses a different N-terminus from MITF-M, but shares the same bHLH/LZ region. The bHLH/LZ regions of TFE3 and TFEC show >85% identity with that of MITF-M. Every combination of LEF-1 either with MITF-A, TFE3 or TFEC was able to activate the DCT gene promoter, whereas c-Myc exerted no noticeable effects (Figure 8B). These results suggest a novel role for MITF/TFE3 proteins as downstream modulators of Wnt signaling. Thus, multiple MITF isoforms and other family members, such as TFE3, may be involved in efficient propagation of Wnt signals in different cell types, depending on the expression levels of a given MITF isoform or other family member.

Discussion

Here we provide evidence for a novel mechanism by which MITF regulates gene transcription through LEF-1, thereby ensuring efficient propagation of Wnt signals in melanocytes. Thus, MITF-M could function in melanocytes as a target, as well as a nuclear effector of Wnt signaling. Moreover, we have shown that functional
cooperation of MITF-M and LEF-1 requires a hitherto unidentified factor that binds the CRE-like motif (−242 to −231) of the DCT promoter (summarized in Figure 9). The functional importance of this 12-bp motif is also supported by the presence of the same sequence at the equivalent position of the mouse DCT gene promoter (Budd and Jackson, 1995). In this context, DCT mRNA expression is upregulated by forskolin, a cAMP-elevating agent, in human melanoma cells (Udono et al., 2001) and mouse melanoma cells (Bertolotto et al., 1998), but not in human retinoblastoma cells (Udono et al., 2001). Future studies will be aimed at exploring the role of cAMP for the observed functional cooperation of MITF-M, LEF-1 and the CRE-like motif-binding protein.

DCT is characterized by its early expression in migrating melanoblasts of mouse embryos (Steel et al., 1992) as well as in certain types of human tumors, such as retinoblastoma (Udono et al., 2001) and glioblastoma (Suzuki et al., 1998). In mouse embryos, the onset of LEF-1 mRNA expression is detected at 7.5 embryonic days (Oosterwegel et al., 1993), which precedes the onset of Mitf expression (−9.5–10.5 days) (Nakayama et al., 1998) and DCT mRNA expression (−10 days) (Steel et al., 1992). Thus, the expression profiles of DCT, LEF-1 and Mitf mRNAs are consistent in part with our proposal that DCT expression is directed by the functional cooperation of MITF-M and LEF-1.

DNLEF-1 could interact with MITF-M, leading to small activation of the DCT promoter (Figures 3B and 4B, lane 9). Thus, β-catenin is required for efficient activation of the DCT promoter, but dispensable for the interaction with MITF-M. This notion is of physiological significance, because even in the absence of Wnt signals, LEF-1 by itself could cooperate with MITF-M to maintain DCT gene transcription. DCT has been considered to play an important role in detoxification of melanin precursors (Steel et al., 1992). Taken together, we assume that Wnt signaling initially induces MITF-M expression, and then the expressed MITF-M cooperates with LEF-1 to initiate and maintain transcription of the DCT gene.

At least two separate regions of LEF-1 are required for the interaction with MITF-M: the middle portion located between the β-catenin-binding domain and the HMG box, and the NLS. The middle portion of LEF-1 is known as the
context-dependent activation domain and is not conserved in TCF-1B. Interestingly, nuclear transport proteins bind to the NLS of LEF-1 but not TCF-1B (Prieve et al., 1998). These results suggest that the middle portion of LEF-1 may profoundly influence the protein-binding potential of LEF-1 NLS.

The observed functional difference between LEF-1 and TCF-1 may account for the complex phenotypes of LEF-1-deficient mice (van Gendersen et al., 1994), compared with TCF-1-deficient mice (Verbeek et al., 1995), despite their overlapping expression profiles during embryonic development (Oosterwegel et al., 1993). The LEF-1−/− mice lack teeth, hair follicles, whiskers (vibrissae) and trigeminal nerves of neural crest origin, and die shortly after birth, whereas TCF-1−/− mice showed T-cell abnormality but appear healthy and are fertile. It is noteworthy that the LEF-1-deficient mice contain unpigmented melanocytes in the skin. Thus, LEF-1 is essential for hair follicle development and melanin production in melanocytes, but dispensable for melanocyte development during embryogenesis. These results suggest that a certain member of the TCF family, such as TCF-1, may be responsible for Mitf-M expression during fetal development of LEF-1-deficient mice. In fact, TCF-1 mRNA is expressed in human epidermal melanocytes (Figure 1A).

The homozygous Mitf−/− mice appear normal when young with uniformly lighter color and congenital white spots, but show ageing-dependent melanocyte loss (Lerner et al., 1986). In addition, plucking hairs promotes the regrowth of amelanotic hairs due to melanocyte loss in the plucked areas. These phenotypes indicate a crucial role of Mitf-M in postnatal maintenance of follicular melanocytes. In fact, expression of Mitf mRNA becomes undetectable in most tissues, except for follicular melanocytes, in which Mitf mRNA expression is maintained even in adult mice (Nakayama et al., 1998). Here we suggest that the mit mutation may impair the formation of a stable complex on the DCT promoter involving MITF-M, LEF-1 and other factors (Figure 9), which leads to the defect in Wnt signal transduction in follicular melanocytes. In contrast, the m mutation does not severely affect such a protein–protein interaction on the DCT promoter, probably due to the property of MITF-Mα protein as non-DNA-binding cofactor for LEF-1. The latter notion is consistent in part with the results that the base changes at the M box, the binding site for MITF-M, resulted in only a 2-fold reduction in the synergistic activation (see Figure 1B).

Materials and methods

RT–PCR analysis

Total RNA was prepared from culture cells and tissues as described (Amae et al., 1998), and the first strand cDNA was synthesized using oligo(dT)12–18 primer. A portion of the reverse transcription mixture was subjected to PCR (35 cycles of 30 s at 95°C, 30 s at 59°C and 2 min at 72°C) using AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The PCR primers used were: 5'-ggggatggTGCTGGCGAGCACTG- CATCC-3' and 5'-ggtacgctGTAGTGGACTGTGCTTC-3' for LEF-1 (Waterman et al., 1991); 5'-GTCACCGACGCGATGCATGC- TGATGC-3' and 5'-CAATCCGCACTGATGCACGTGTCAGATG-3' for TCF-1 (van de Wetering et al., 1996); and 5'-CCACCGGACGACCAAATCCGATGCA-3' and 5'-CTTAGCGCCGAGCTGAAGTGCCAC-3' for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower cases in these primers indicate additional nucleotides.

Plasmid construction

MITF expression plasmids, pUC/CMV-MITF-M and pUC/CMV-MITF-A, were described previously (Yasumoto et al., 1994; Amae et al., 1998). FL9B, a mammalian expression plasmid, contains the full-length human LEF-1 cDNA (Waterman et al., 1991). TCF-1B cDNAs were gifts from H. Clevers (van de Wetering et al., 1996). Reporter plasmids contain the firefly luciferase gene, linked to the 5′-flanking region of the human DCT gene (Yokoyama et al., 1994). pENL, a β-galactosidase expression vector, was used as an internal control for the transfection efficiency. LEF-1 cDNA and its deletion mutants were inserted into pEGFP-N1, encoding enhanced EGFP (Clontech) for the analysis of subcellular localization. These LEF-1 proteins are fused to GFP at their C-termini. LEF-1–TCF-1 chimeric cDNAs were constructed by replacing the C-terminal portion of LEF-1 by that of TCF-1. Detailed procedures for construction of various DCT reporter plasmids, truncated LEF-1 mutants and chimeric proteins are available on request. Human c-Myc cDNA was a gift from M.Obinata and was cloned in a mammalian expression vector pRc/CMV (Invirotegen). All constructions were confirmed by sequencing.

Cell cultures and transfection

HeLa human uterine cervical cancer cells and COS-7 monkey kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). Melan-a murine immortalized melanocytes were grown in Minimum Essential Medium supplemented with 10% fetal calf serum and 200 mM phenol 12-myristate 13-acetate (Bennett et al., 1987). HeLa cells were transfected with each fusion plasmid and a β-galactosidase expression plasmid by the calcium phosphate precipitation method (Yasumoto et al., 1997). The amount of reporter DNA was kept at 4 μg and the total amount of DNA was kept constant (usually 9.4 μg/60-mm dish). At 24 h post-transfection, cells were harvested, and luciferase activity was measured with a Picogreen luciferase assay system (Toyo Ink) and a Lumat LB9507 (Berthold). The luciferase activity was normalized with each β-galactosidase activity that represents an internal control. The magnitude of activation is presented as the ratio of normalized luciferase activity and that with a vector DNA. The results of at least three independent experiments are shown with standard deviations. Melan-a cells and COS-7 cells were transfected using FuGENE 6 transfection reagent (Roche).

HeLa cells were transfected with an equal amount of LEF-1 and each bHLH/LZ protein construct (2 μg each), and the total amounts of plasmid DNA were maintained at 4 μg with the vector DNA (pRc/CMV). Expression vectors for TFII (Beckmann et al., 1990) and TFE3 were constructed as described previously (Yasumoto and Shibahara, 1997). The data are presented as the ratio of normalized luciferase activity obtained with each combination and with vector DNA.

EMSA

Nuclear extracts were prepared from HeLa cells untransfected or transfected with MITF-M and LEF-1 by the method of Schreiber et al. (1989). EMSA was performed as described previously (Yasumoto et al., 1995). Except that the binding reaction contained 0.5 M HEPES–NaOH pH 7.9, 80 mM KCl, 0.6 mM EDTA, 0.6 mM EGTA, 12% glycerol, 2 mM MgCl2 and 0.1 mg/ml poly(dIdC). GST–LEF-1 fusion protein was prepared as described previously for the GST–MITF-M fusion protein (Yasumoto et al., 1995) and used for EMSA.

In vitro protein–protein interactions

In vitro binding studies were performed using GST–MITF immobilized on the GST–Sepharose resin and COS-7 nuclear extract containing c-Myc-tagged LEF-1. COS-7 cells (5 × 106) were transfected with 8 μg of a LEF-1 expression vector and harvested 42 h post-transfection. GST–MITF fusion proteins were purified on GST– Sepharose 4B resin (Amersham-Pharmacia), according to the manufacturer’s instructions. The resin was preincubated with non-transfected COS-7 nuclear extract. The resulting streptavidin–biotinylated GST–MITF fusion protein was washed with 200 μl of buffer C four times, and a final suspension of 10 μl was applied to SDS–PAGE. c-Myc-tagged LEF-1 was detected by western blot analysis with anti-c-Myc antibody (Santa Cruz Biotechnology).
Yeast two-hybrid assay
Yeast two-hybrid assay was performed using MATCHMAKER Two-Hybrid System 2 (Clontech), according to the manufacturer’s instructions. LIF-1 was chosen as a bait, because LIF-1 itself does not act as a transcriptional activator in yeast cells (Prieve et al., 1998) and a strong transcription activation domain is located near the N-terminal region of MITF-M (Sato et al., 1997). LIF-1 cDNA and portions of MITF-M cDNA fragments were inserted into pAS2-1, encoding the GAL4 DNA-binding domain, and pACT2, encoding the GAL4 transactivation domain, respectively.

Mammalian two-hybrid assay
To assess the effect of mutation in the 9HLH/LZ region of MITF-M on the interaction with LIF-1, we performed mammalian two-hybrid assay using Mammalian MATCHMAKER Two-Hybrid Assay Kit (Clontech). The DNA segment, containing five consensus GAL4-binding sites and an adenovirus E1b minimal promoter region, was isolated from pG5CAT vector and inserted into the multiple cloning site of pGL3-Basic (Promega), generating a reporter plasmid pGL3-G5P. Full-length human LIF-1 cDNA was inserted in the pCM cloning vector, generating pCM-LIF-1 that codes for the LIF-1 fused to GAL4 DNA-binding domain. The 9HLH/LZ region (amino acids 138–372) of MITF-M or its mutant (MITF-M9 or MITF-M6) was fused to an activation domain derived from the VP16 protein of herpes simplex virus. The blunted-ended BamHI fragment, containing each 9HLH/LZ region, was inserted at the blunt-ended SalI site of pVP16 vector. The resulting plasmids pVP-M-iw-B, pVP-M-w-B and pVP-M-b-B encode the fusion proteins MITF-M-B, MITF-M-iw-B and MITF-M-w-B, respectively (see Figure 7C). 293 human embryonic kidney cells were grown in Eagle’s medium α-modification supplemented with 10% FBS. 293 cells were transfected using LipofectAMINE 6 transfection reagent. The amount of total DNA was kept at 4 μg per 60 mm dish (1 μg of reporter and 1.5 μg each of effector DNA). Transfected cells were then incubated for 43 h at 37°C, and were harvested for luciferase assay.

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